Instructions for Use

For rapid, sensitive and accurate detection of Factor Xa activity.

This product is for research use only and is not intended for diagnostic use.
# Table of Contents

## INTRODUCTION
1. BACKGROUND 2
2. ASSAY SUMMARY 3

## GENERAL INFORMATION
3. PRECAUTIONS 4
4. STORAGE AND STABILITY 4
5. LIMITATIONS 4
6. MATERIALS SUPPLIED 5
7. MATERIALS REQUIRED, NOT SUPPLIED 5
8. TECHNICAL HINTS 6

## ASSAY PREPARATION
9. REAGENT PREPARATION 7
10. STANDARD PREPARATION 8
11. SAMPLE PREPARATION 10

## ASSAY PROCEDURE and DETECTION
12. ASSAY PROCEDURE and DETECTION 11

## DATA ANALYSIS
13. CALCULATIONS 13
14. TYPICAL DATA 14

## RESOURCES
15. QUICK ASSAY PROCEDURE 15
16. TROUBLESHOOTING 16
17. INTERFERENCES 17
18. FAQ 18
1. **BACKGROUND**

Factor Xa Activity Assay Kit (Fluorometric) (ab204711) utilizes the ability of Factor Xa to cleave a synthetic substrate thereby releasing a fluorophore, AMC, which can be quantified by fluorescence readers. This assay kit is simple, rapid and can detect Factor Xa activity as low as 1 ng.

Factor Xa (FXa) is the activated form of the coagulation factor X (Stuart-Power factor, thrombokinase, prothrombinase, thromboplastin, E.C.3.4.21.6). Factor X, a serine endopeptidase plays an important role at several stages of the coagulation pathway. It acts by converting prothrombin into active thrombin by complexing with activated co-factor V in the prothrombinase complex. Unfractionated heparin and various low molecular weight heparins bind to plasma cofactor antithrombin to inactivate several coagulation factors including factor Xa.
INTRODUCTION

2. ASSAY SUMMARY

- Standard Curve Preparation

  ↓

- Sample Preparation

  ↓

- Add Reaction Mix

  ↓

- Measure fluorescence (Ex/Em = 350/450 nm) in a kinetic mode at 37°C for 30-60 minutes*

*For kinetic mode detection, incubation time given in this summary is for guidance only.
3. **PRECAUTIONS**

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. **STORAGE AND STABILITY**

Store kit at -20°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature. **Reconstituted components are stable for 2 months.**

5. **LIMITATIONS**

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not use kit or components if it has exceeded the expiration date on the kit labels.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.
6. MATERIALS SUPPLIED

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount</th>
<th>Storage Condition (Before Preparation)</th>
<th>Storage Condition (After Preparation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FXa Dilution Buffer</td>
<td>1 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>FXa Assay Buffer</td>
<td>15 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>FXa Enzyme Standard</td>
<td>5 µL</td>
<td>-20°C</td>
<td>-80°C</td>
</tr>
<tr>
<td>FXa Substrate</td>
<td>200 µL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
</tbody>
</table>

7. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microcentrifuge
- Pipettes and pipette tips
- Fluorescent microplate reader – equipped with filter Ex/Em = 350/450 nm
- 96 well plate with clear flat bottom preferably white
- Heat block or water bath
8. **TECHNICAL HINTS**

- This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.

- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.

- Keep enzymes, heat labile components and samples on ice during the assay.

- Make sure all buffers and solutions are at room temperature before starting the experiment.

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.

- Avoid foaming or bubbles when mixing or reconstituting components.

- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.

- Ensure plates are properly sealed or covered during incubation steps.

- Make sure you have the right type of plate for your detection method of choice.

- Make sure the heat block/water bath and microplate reader are switched on.
9. **REAGENT PREPARATION**

- Briefly centrifuge small vials at low speed prior to opening.

9.1 **FXa Assay Buffer:**
Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

9.2 **FXa Dilution Buffer:**
Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

9.3 **FXa Enzyme Standard:**
Prepare a stock solution of FXa Enzyme (100 ng/μL) by adding 45 μL of FXa Dilution buffer to the 5 μL of FXa Enzyme Standard provided in the vial. Mix well by pipetting up and down. Aliquot enzyme so that you have enough volume to perform the desired number of assays. Avoid repeated freeze/thaw. Store at -80°C. Keep on ice while in use.

9.4 **FXa Substrate:**
Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.
10. STANDARD PREPARATION

- Always prepare a fresh set of standards for every use.
- Diluted standard solution is can be stored at 4°C. Use within one week.

10.1 Prepare 200 µL of 5 ng/µL FXa Enzyme Standard by diluting 10 µL of the diluted FXa Enzyme stock solution (100 ng/µL) with 190 µL of FXa Dilution Buffer.

10.2 Using 5 ng/µL FXa Enzyme Standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes.

<table>
<thead>
<tr>
<th>Standard #</th>
<th>Volume of Standard (µL)</th>
<th>Assay Buffer (µL)</th>
<th>Final volume standard in well (µL)</th>
<th>End Conc FXa in well (ng/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>150</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>138</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>126</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>36</td>
<td>114</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>5</td>
<td>48</td>
<td>102</td>
<td>50</td>
<td>80</td>
</tr>
<tr>
<td>6</td>
<td>60</td>
<td>90</td>
<td>50</td>
<td>100</td>
</tr>
</tbody>
</table>

Each dilution has enough amount of standard to set up duplicate readings (2 x 50 µL).
If a more sensitive assay is required:

10.3 Prepare a 0.5 ng/µL FXa Enzyme Standard by diluting 20 µL of the 5 ng/µL FXa Enzyme Standard (Step 10.1) with 180 µL of FXa Dilution Buffer.

10.4 Using 0.5 ng/µL FXa Enzyme Standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes.

<table>
<thead>
<tr>
<th>Standard #</th>
<th>Volume of Standard (µL)</th>
<th>Assay Buffer (µL)</th>
<th>Final volume standard in well (µL)</th>
<th>End Conc FXa Enzyme in well (ng/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>150</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>138</td>
<td>50</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>126</td>
<td>50</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>36</td>
<td>114</td>
<td>50</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>48</td>
<td>102</td>
<td>50</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>60</td>
<td>90</td>
<td>50</td>
<td>10</td>
</tr>
</tbody>
</table>

Each dilution has enough amount of standard to set up duplicate readings (2 x 50 µL).
ASSAY PREPARATION

11. SAMPLE PREPARATION

General Sample information:

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step before storing the samples. Alternatively, if that is not possible, we suggest that you snap your samples in liquid nitrogen upon extraction and store the samples immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

11.1 Plasma:

Plasma samples can be tested directly by adding sample to the microplate wells.

11.2 Purified protein:

Dilute purified protein in FXa Assay buffer before adding directly to the microplate wells.

NOTE: To find the optimal values and ensure your readings will fall within the standard values, we recommend performing several dilutions of the sample.
12. ASSAY PROCEDURE and DETECTION

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- It is recommended to assay all standards, controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.

12.1 Set up Reaction wells:
- Standard wells = 50 µL standard dilutions.
- Sample wells = 2 – 50 µL samples (adjust volume to 50 µL/well with FXa Assay Buffer).

12.2 Reaction Mix:
Prepare 50 µL of Reaction Mix for each reaction:

<table>
<thead>
<tr>
<th>Component</th>
<th>Reaction Mix (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FXa Assay Buffer</td>
<td>48</td>
</tr>
<tr>
<td>FXa Substrate</td>
<td>2</td>
</tr>
</tbody>
</table>

Mix enough reagents for the number of assays (samples and controls) to be performed. Prepare a master mix of the Reaction Mix to ensure consistency. We recommend the following calculation: X µL component x (Number reactions +1)

12.3 Add 50 µL of Reaction Mix into each standard and sample well. Mix well.

12.4 Measure output on a fluorescent microplate reader at Ex/Em = 350/450 nm in a kinetic mode, every 2 – 3 minutes, for 30-60 minutes at 37°C protected from light.
To reduce the background from sample, fluorescence can be read at Ex/Em = 350/460 nm or Ex/Em = 350/470 nm. However, the sensitivity may be lower at these wavelengths.
NOTE: Sample incubation time can vary depending on Factor Xa (FXa) activity in the samples. We recommend measuring fluorescence in kinetic mode and then choosing two time points ($T_1$ and $T_2$) during the linear range.

RFU value at $T_2$ should not exceed the highest RFU in the standard curve.
13. **CALCULATIONS**

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).

13.1 Average the duplicate reading for each standard and sample.

13.2 Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.

13.3 Plot the corrected absorbance values for each standard as a function of the final concentration of FXa Enzyme.

13.4 Draw the best smooth curve through these points to construct the standard curve. Calculate the trend line equation based on your standard curve data (use the equation that provides the most accurate fit).

13.5 Use the $\Delta RFU_{350/450nm}$ to obtain $B$ (in ng) of Factor Xa.

13.6 Activity of Factor Xa in the test is calculated as:

$$FXa \ Activity = \left( \frac{B}{V} \right) \times \text{Dilution Factor} = \text{ng/mL} = \mu g/L$$

Where:

- $B$ = Amount of FXa from Standard Curve (ng).
- $V$ = Original sample volume added into the reaction well (mL).
14. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.

Figure 1. Standard plot of FXa activity measured at two different emission wavelengths (450 and 460 nm) keeping the excitation at 350 nm.

Figure 2. FXa activity was measured in plasma samples in the presence and absence of a FXa inhibitor, GGACK Dihydrochloride. S = Substrate, I = Inhibitor.
15. QUICK ASSAY PROCEDURE

**NOTE:** This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

- Prepare standard and prepare enzyme mix; get equipment ready.
- Prepare appropriate standard curve.
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings).
- Set up plate for standard (50 µL) and samples (50 µL) wells.
- Prepare Factor Xa Reaction Mix (Number samples + standards + 1).

<table>
<thead>
<tr>
<th>Component</th>
<th>Reaction Mix (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FXa Assay Buffer</td>
<td>48</td>
</tr>
<tr>
<td>FXa Substrate</td>
<td>2</td>
</tr>
</tbody>
</table>

- Add 50 µL of Factor Xa Reaction Mix to the standard and sample wells.
- Incubate plate at 37°C during 30-60 minutes and read fluorescence at Ex/Em= 350/450 nm or Ex/Em = 350/470 nm in a kinetic mode.
## 16. Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay not working</td>
<td>Use of ice-cold buffer</td>
<td>Buffers must be at room temperature</td>
</tr>
<tr>
<td></td>
<td>Plate read at incorrect wavelength</td>
<td>Check the wavelength and filter settings of instrument</td>
</tr>
<tr>
<td></td>
<td>Use of a different 96-well plate</td>
<td>Clear plates</td>
</tr>
<tr>
<td>Sample with erratic readings</td>
<td>Presence of interfering substance in the sample</td>
<td>Check protocol for interfering substances; deproteinize samples</td>
</tr>
<tr>
<td>Lower/Higher readings in samples and Standards</td>
<td>Improperly thawed components</td>
<td>Thaw all components completely and mix gently before use</td>
</tr>
<tr>
<td></td>
<td>Allowing reagents to sit for extended times on ice</td>
<td>Always thaw and prepare fresh reaction mix before use</td>
</tr>
<tr>
<td></td>
<td>Incorrect incubation times or temperatures</td>
<td>Verify correct incubation times and temperatures in protocol</td>
</tr>
<tr>
<td>Standard readings do not follow a linear pattern</td>
<td>Pipetting errors in standard or reaction mix</td>
<td>Avoid pipetting small volumes (&lt; 5 µL) and prepare a master mix whenever possible</td>
</tr>
<tr>
<td></td>
<td>Air bubbles formed in well</td>
<td>Pipette gently against the wall of the tubes</td>
</tr>
<tr>
<td></td>
<td>Standard stock is at incorrect concentration</td>
<td>Always refer to dilutions on protocol</td>
</tr>
<tr>
<td>Unanticipated results</td>
<td>Measured at incorrect wavelength</td>
<td>Check equipment and filter setting</td>
</tr>
<tr>
<td></td>
<td>Samples contain interfering substances</td>
<td>Troubleshoot if it interferes with the kit</td>
</tr>
<tr>
<td></td>
<td>Sample readings above/ below the linear range</td>
<td>Concentrate/ Dilute sample so it is within the linear range</td>
</tr>
</tbody>
</table>
17. INTERFERENCES

These chemicals or biological materials will cause interferences in this assay causing compromised results or complete failure:

- **RIPA buffer** – it contains SDS which can destroy/decrease the activity of the enzyme.
18. FAQ